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DIAGNOSIS OF AIDS-RELATED INTESTINAL PARASITES

ANNUAL REPORT

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ABSTRACT

Cryptosporidium, Giardia lamblia and Entamoeba histolytica are all protozoan parasites which infect the gastrointestinal tract of humans, are known agents of diarrhea, and are difficult to diagnose using traditional parasitologic techniques. Cryptosporidium causes a life-threatening, so far untreatable, diarrheal illness in HIV-infected patients, of whom approximately 4% are infected in the United States. Giardia lamblia and Entamoeba histolytica, for which excellent treatment modalities are available, may infect some groups of HIV patients more than other individuals.

During the first year of this project, a double antibody sandwich enzyme immunoassay (ELISA) to detect Cryptosporidium antigen in fecal specimens was developed; it detected (1) between 1000 and 10,000 purified Cryptosporidium oocysts and (2) specific antigen in fecal specimens from 14 of 15 infected patients. During the second year, sensitivity and specificity data will be generated using fecal specimens from large numbers of individuals with cryptosporidiosis. *Keywords:* - HIV, ELISA, Cryptosporidium

Sero-epidemiologic studies using a previously developed ELISA to detect specific IgG antibodies to Cryptosporidium were completed as follows: (1) In Lima, Peru, 64% (267/415) of children and adults surveyed had detectable specific IgG antibody suggesting that infection sometime in life is common for this group and supporting the notion that there are regions of the world highly endemic for Cryptosporidium. (2) In U.S. Peace Corps volunteers, 32% (24/75) had detectable anti-Cryptosporidium IgG before leaving the U.S. and approximately 14% per year of seronegative individuals became seropositive after one or two years of Peace Corps service in Africa. This implies the risk of infection is real for travelers and temporary workers in highly endemic areas. (3) In a retrospective evaluation of a presumed waterborne outbreak of Cryptosporidium-associated diarrhea, between 55% and 77% of residents had detectable specific IgG 2 weeks or more after any symptoms. This was in comparison to 35% of healthy Centers for Disease Control employees (similar to healthy Peace Corps volunteers). Serologic evaluation helped confirm that Cryptosporidium was the agent of diarrhea in this waterborne outbreak affecting approximately 13,000 individuals.

Studies were also begun to assess the protective ability of breast-milk antibody against Cryptosporidium infection in animal models. Preliminary data suggests that protection occurs using hyperimmune bovine colostrum in a calf model; during the second year of this project, the calf study will be completed, further studies initiated using a new mouse model, and if promising, compassionate use considered for HIV-infected patients.

Previously developed ELISA tests to detect antigens of Giardia lamblia or Entamoeba histolytica in fecal specimens of infected persons were restandardized and used in a study of etiology of diarrhea in foreigners in Nepal and in a study on detection and treatment of giardiasis in Peru.

Objective I. Development of an ELISA to detect Cryptosporidium antigens in fecal specimens.

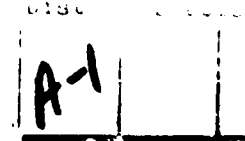
A. Background and Statement of Problem

Cryptosporidium is now commonly accepted as a causative agent of diarrhea in immunologically healthy, as well as immunocompromised individuals with at least 3.6% of AIDS patients infected (1,2). While immunologically healthy patients recover spontaneously within 30 days of onset of symptoms, their clinical illness can be severe and oocyst shedding and potential transmission persistent for as long as 60 days after symptoms remit (3,4,5). Immuno-compromised persons, including patients with malignancies, with malnutrition or with certain concomitant illnesses such as measles or chicken pox, may be susceptible to more severe clinical manifestations with cure only when the cause of immunosuppression is removed (6,7,8,9,10). Treatment remains enigmatic: more than 50 therapeutic modalities have been unsuccessful (1). One anti-microbial agent, spiramycin, currently in clinical trial in the U.S. and in use in some overseas locations may alleviate clinical symptoms although not achieve parasitologic cure (Juan-Carlos Wirtz, Facultad de Medicina, Universidad Chile, Santiago, Chili; Rosemary Soave, New York Hospital, Cornell University School of Medicine, personal communications). Hyperimmune bovine colostrum has also recently been associated with remission of clinical symptoms in humans (11).

Evidence for the ubiquity of Cryptosporidium infection continues to accumulate with human infection reported from more than 35 countries on six continents. Based on examination of single fecal samples for Cryptosporidium oocysts generally between 2 and 4% of those examined in Europe and North America and between 3 and 10% of those examined in Africa, Asia, Australia, and Central/South America are found infected (1). This suggests a higher frequency of Cryptosporidium infection in economically less developed areas of the world.

Cryptosporidium is acquired through oral ingestion. Animal-to-person transmission (pets, farm and laboratory animals), person-to-person transmission (family members, sexual partners, day-care center attendees, medical caretakers), and waterborne transmission (travelers, drinking water contaminated by contact with sewage disposal systems, or by water treatment processes inadequate to remove the parasite from surface supplies) are thought common. In one recent waterborne outbreak in the U.S., an estimated 13,000 individuals became symptomatic after drinking water purified to meet all existing standards (12).

Diagnosis still rests on identification of oocysts in fecal specimens using one of more than fifteen described procedures (1). Proficiency with these diagnostic techniques, in use for only a few years in most clinical laboratories, varies, as does over-all accuracy; recently oocysts have been described which do not stain using the modified acid fast staining technique, up to now one of the most widely used diagnostic techniques (13). Accurate diagnosis remains important in identifying individuals who may be transmitting Cryptosporidium to others, in identifying (through epidemiologic studies) geographic areas where Cryptosporidium infection is likely endemic or areas where outbreaks of cryptosporidiosis may be occurring, and ultimately, in



evaluating efficacy of newer treatments. An ELISA to detect Cryptosporidium antigen in fecal specimens continues to offer a simple, rapid, cost-effective and easily standardized diagnostic tool; its potential military utility both in care of AIDS patients and in care of immunologically healthy troops is self-evident.

B. Results to Date

Initial studies were performed using previously prepared anti-Cryptosporidium rabbit polyclonal antisera and monoclonal antibodies in a double antibody indirect ELISA. After checkerboard titration to determine optimal reagent concentrations, Cryptosporidium oocysts were purified from calf feces (14) counted and used as test antigen. Between 1000 and 10,000 oocysts were detected, proving system feasibility.

Because of the small quantity of specific reagents actually available, additional polyclonal antisera have been produced. Colostrum-deprived calves were orally infected with Cryptosporidium oocysts and fecal output collected (courtesy Ronald Fayer). Oocysts were purified using flotation in saturated NaCl, with subsequent sedimentation in distilled water, washing in sodium hypochlorite, and sonication. Rabbits and goats were screened for specific anti-Cryptosporidium antibodies and animals without detectable antibodies immunized with purified Cryptosporidium over a four month period to produce high titered antisera.

Using goat and rabbit polyclonal antisera, a double antibody indirect ELISA was again performed; checkerboard titrations have so far shown that a 1:10,000 dilution of specific rabbit antisera can be used to coat microtiter plates with a second specific goat antibody used in a 1:400 dilution. Using stool specimens from at least 15 individuals with known Cryptosporidium infections, so far 14/15 have had significantly higher ELISA O.D. readings than control specimens from individuals without Cryptosporidium.

C. Future Directions

Refinement of this ELISA includes (1) additional optimization using other specific antibody combinations; (2) standardization to include control microtiter plate wells coated with non-immune serum; (3) identification of a panel of Cryptosporidium negative stool specimens to be used as negative controls on each microtiter plate for interplate standardization of results; (4) continued testing of stool specimens with and without Cryptosporidium to establish sensitivity and specificity for this ELISA. Plans for field testing this ELISA both in Peru (known to have a high prevalence and incidence of Cryptosporidium infection) and in Zambia (where a collaborative study between USUHS and the Ministry of Health on clinical features of AIDS is underway) are in process.

D. Additional Results Pertinent to Cryptosporidium Infections in Humans.

1. Seroepidemiologic studies. Using a previously developed ELISA to detect anti-Cryptosporidium IgM or IgG (14), work was completed assessing seroprevalence of Cryptosporidium in Peru and Venezuela in collaboration with Robert H. Gilman (Department of International Health, Johns Hopkins University School of Public Health and Hygiene, Baltimore, Maryland and Universidad Peruana Cayetano Heredia, Lima, Peru), Claudio F. Lanata (Instituto de Investigacion Nutricional, Lima, Peru) and Irene Perez-Schael (Instituto de Biomedicina, Caracas, Venezuela) (15). The ELISA was used to examine randomly selected sera from 389 children and adults in Lima, Peru, and 84 children in Maracaibo and Caracas, Venezuela. In Peru, 19.8%, and in Venezuela, 15.5% of the study population was positive for specific IgG and IgM simultaneously, consistent with active or recent infection, and representing a larger percent than normally reported from stool examinations of individuals seeking medical attention. Sixty-four percent from each country had detectable anti-Cryptosporidium IgG, suggesting in economically disadvantaged regions such as these, the majority of residents have been infected sometime in life. Detection of specific IgG increased in the 2-3 year-old group, indicating this is a common age for infection. Persistence of IgG and less often, IgM antibody response occurred over 12 months in some individuals, although whether or not this protects from or modifies the nature of the infection or symptoms needs further investigation. These findings suggest that Cryptosporidium infections are endemic in the communities surveyed and that most residents have been infected.

Using the same ELISA test in collaboration with Thomas Nutman (Laboratory of Parasitic Diseases, NIH) and Maurita Mulligan (U.S. Peace Corps, Washington, D.C.), sera from 75 U.S. Peace Corps volunteers were examined in an effort to define the prevalence of Cryptosporidium infection in healthy U.S. adults, and to determine how often Cryptosporidium infection occurs after relocation to a situation of potentially great exposure (manuscript submitted). 32% of volunteers had detectable anti-Cryptosporidium IgG initially, suggesting that in the United States as well, infection frequently occurs. After 6 weeks, 1 year or two years overseas, 5%, 14% and 13.6% of seronegative individuals respectively became newly IgG positive. Of 22 volunteers followed for two years, only 2 showed no specific immune response to Cryptosporidium during this time. This implies that the risk of infection is real for travellers and temporary workers in highly endemic areas.

Finally, at the request of the Centers for Disease Control in conjunction with their retrospective evaluation of a presumed waterborne outbreak of Cryptosporidium-associated diarrhea in Carrollton County, Georgia, serum specimens were evaluated from (1) 20 presumed healthy CDC employees; (2) 39 Carrollton County residents with gastrointestinal symptoms and Cryptosporidium detected on stool examination; (3) 37 symptomatic Carrollton County residents with no Cryptosporidium organisms detected on stool examination; and (4) 20 asymptomatic and 78 symptomatic Carrollton County residents without any fecal examinations. IgM and IgG antibody to Cryptosporidium were simultaneously detected more often in symptomatic Carrollton County residents (14.6%) than in asymptomatic residents (5%) or presumed healthy CDC employees (5%), consistent with recent infection in the symptomatic individuals. For residents actively excreting oocysts, 76.9% had specific IgG 14 or more days after onset of symptoms, but only 27.2% earlier than 14 days; for residents not excreting oocysts, 55% had specific IgG irrespective of time from onset of symptoms. IgG

antibody to Cryptosporidium was detected in 35% of the healthy CDC employees, a percentage comparable to the healthy Peace Corps volunteers. These serologic studies helped confirm the likelihood that Cryptosporidium was the agent of diarrhea in this outbreak (manuscript in preparation). They further suggest Cryptosporidium infections may be more common than realized in the U.S. with potential pockets of endemnicity. (In Carrollton County, the surface water supply to the water treatment plant was likely contaminated by infected cattle; the water treatment plant was basically meeting all water quality standards which were inadequate to remove the Cryptosporidium, circumstances probably duplicated elsewhere).

2. Protective Ability of Breast-milk Antibody in Animal Models. Recently the possibility that hyperimmune bovine colostrum may be therapeutically useful in humans at least in accelerating clinical improvement if not achieving parasitologic cure has been suggested (11). We have participated in two studies evaluating ability of colostrum to protect against Cryptosporidium infection in two animal species. In one set of experiments using a mouse model, mice suckling from dams hyperimmunized both orally and parenterally with Cryptosporidium parvum oocysts were not protected from infection. Immune dams produced serum antibody against C. parvum while non-immune control dams did not; anti-Cryptosporidium IgA and IgG were demonstrated in milk whey extracted from the stomachs of mice suckling immune dams but not in milk whey from mice suckling non-immune dams (16). In a second set of on-going experiments, pregnant cows were immunized by both the parenteral and intra-mammary routes with C. parvum oocysts. Colostrum harvested after delivery had high titers of specific anti-Cryptosporidium IgG-1, IgA and IgM and is currently be used to assess protective ability (Ronald Fayer, Animal Parasitology Institute, USDA, Beltsville, MD).

Objective II. Conversion of conventional ELISA systems for detection of antigens in fecal specimens for Cryptosporidium, Giardia lamblia, or Entamoeba histolytica.

A. Background and Statement of problem

Since the problem was stated in the original proposal, little progress has been made in developing new and simpler rapid techniques for the diagnosis of either intestinal amebiasis or giardiasis. Conventional ELISA tests to detect antigens of Entamoeba histolytica or Giardia lamblia in fecal specimens are now truly well-established and have been developed by at least six groups for E. histolytica and three for G. lamblia (17,18,19,20,21,22,23,24,25) substantiating efficacy. The Giardia assay has been shown to detect antigens which are shared in common by at least eight distinct Giardia isolates (26). In one recent application, ELISA analysis of stool specimens obtained after oral inoculation of human volunteers with Giardia lamblia showed that 94.5% of those with cysts in their stools were positive by ELISA and that during treatment, the ELISA was significantly better than fecal examination in detection of Giardia lamblia antigen (27). Although two commercial companies have taken preliminary steps toward producing marketable ELISA kits for each parasite, conversion of existing systems to technology simpler tests remains a pre-requisite to their utility outside a research laboratory setting.

B. Results to Date

1. Entamoeba histolytica ELISA. Reagents for the ELISA previously developed were obtained (20). Optimal concentrations of reagents were retested by checkerboard titration since the commercially-available specific monoclonal antibody had been modified since last used. Sensitivity and specificity were re-established using the parasitologically-known stool specimens, frozen at -70°C.

The ELISA assay was used in an on-going study on the etiology of diarrheal disease among travelers, foreign residents and Peace Corps Volunteers in Nepal (through David N. Taylor, M.D., Peter Echeverria, M.D., AFRIMS, Bangkok, Thailand and Robin Houston, M.D., U.S. Peace Corps, Kathmandu, Nepal). More than 300 stool specimens from patients at two clinic locations were examined by microscopy in the field and by ELISA at USUHS after specimens were shipped frozen at -70°C. About 5% of patients from each clinic had ELISA detectable E. histolytica antigen, compared to 4% by microscopy at one clinic and 26% at another clinic thought to have significant problems in misdiagnosis. The correlation between microscopy and ELISA was approximately 50% at the first clinic suggesting that the ELISA may be a reasonable alternative diagnostic technique which should be tested directly in the field (manuscript submitted).

2. Giardia lamblia ELISA. Production of new specific polyclonal antibodies in goats and rabbits using cultured Giardia lamblia trophozoites is underway.

Field testing of the ELISA test originally developed, using scant remaining reagents, was completed in Lima, Peru in collaboration with Robert Gilman, M.D. (affiliation listed above) and Homero Martinez, M.D., Instituto Nacional de la Nutricion, Mexico City. In this study, 53 children with at least one microscopic stool examination positive for Giardia lamblia were identified, randomized to a control group and a group treated with tinidazol, and followed with repeat stool examinations at two to three days and five to six days. All stool specimens were examined microscopically by at least four techniques and by ELISA performed in the field. Although final analysis of results is still in process, preliminary results show that Giardia antigen was identified by ELISA significantly more often than by microscopy in the first follow-up specimens, and the treatment group was negative by ELISA significantly more often than the control group. Using microscopic examination as the gold standard, sensitivity of the ELISA in the field was at least 90% (manuscript in preparation).

C. Future Directions

Once the new reagents are available for the Giardia lamblia ELISA, this test will be restandardized and sensitivity and specificity data re-established. All three antigen detection ELISA systems will then be converted to technically simpler systems, either as originally outlined or using nitrocellulose "DOT-blot" technology. In addition, plans are underway to begin field testing the Entamoeba histolytica ELISA in Mexico (in collaboration with Homero Martinez, M.D., affiliation listed above). Once all reagents are available, both conventional ELISA tests will also be performed in the Venereal Disease-AIDS clinical laboratory at University Teaching Hospital, Lusaka, Zambia in conjunction with the collaborative USUHS AIDS project.

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